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Patent Application of

Philip G. Cavanaugh

for

TITLE OF INVENTION:

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~~METHOD FOR THE DETECTION AND MEASUREMENT OF HAPTEN-CONJUGATED BINDINGENTITIES BY WESTERN AND DOT-BLOT USING ANTI-HAPTEN ANTIBODIES.~~

METHOD FOR THE MEASUREMENT OF BIOLOGICAL LIGAND BINDING BY DETECTION AFTER SECONDARY IMMOBILIZATION.

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CROSS REFERENCE TO RELATED APPLICATIONS: None

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT: This invention was not directly supported by any federally

28 sponsored research.

REFERENCE TO SEQUENCE LISTING, TABLES, OR COMPUTER PROGRAM LISTINGS: None

BACKGROUND OF THE INVENTION

1. Frequently, researchers desire to analyze the ability of various proteins and other factors to bind to cell surfaces. Usually, the type of binding studied is one where the binding factor (ligand) recognizes and binds to a specific receptor for it on the cell surface. Thus, these types of studies are used to examine the inherent properties of the ligand itself, but also are used solely to study the receptor. Analysis of ligand binding to cell surfaces is usually performed directly, wherein that ligand itself is obtained in pure form and is radiolabeled. Usually, ligands are radiolabeled with ^{125}I. More rarely, they are purchased labeled with ^{3}H or ^{14}C. The labeled ligand is assessed for its maintenance of activity, and for its specific (cpm per unit of weight) radioactivity. To measure binding, the radiolabeled material is applied under established optimal conditions to desired cells of known density (cells/unit volume or cell protein/unit volume). Typically, various concentrations (from high to low) of the ligand are added to separate tubes or dishes of cells. Certain cell containers at each dose tested also receive an excess of unlabeled pure ligand. Usually, these excesses are 10 – 200 fold times the concentration of labeled ligand. After the desired binding time has passed, the unbound material from all samples is saved and the cells are washed free of all unbound labeled and unlabeled ligand. The cells are then placed into counting tubes and counted for radioactivity. Initial unbound material is counted also. The amount of labeled ligand bound or unbound is calculated from the known specific cpm. Counts obtained from unlabeled excess ligand-receiving samples are subtracted from the counts obtained from samples treated

with like-dose labeled ligand only. This provides specific cpm bound. The weight amount of specific labeled ligand bound is calculated from the known specific cpm per unit weight. Knowing the cell density, one can calculate amount of specific ligand bound

4 per cell at each ligand dose level. Usually, the data is plotted as specific ligand bound/ ligand unbound/ unit of cells on the y axis and specific ligand bound/unit of cells on the x axis. This produces data with a negative slope and the x-intercept is the maximum amount of ligand able to bind. Therefore, the x-intercept also represents the

8 receptors/cell for the ligand. This type of analysis is referred to as a Scatchard analysis. (Seacchi et. al., 1988; Inoue et. al., 1993; LaGrange et. al., 1993; Gordon, 1995; Cavanaugh and Nicolson, 1998; Cavanaugh et. al., 1999).

12 2. An alternative method to determine ligand binding to cells is to conjugate a particular fluorescent molecule to the pure ligand. Fluorescent labeled material is allowed to bind to cells at various concentrations with or without the presence of unlabeled ligand. After binding is complete, all unbound ligand is washed off and the

16 fluorescence of the cells is determined using a fluorescent spectrophotometer or a fluorescent activated cell sorter instrument (Gordon, 1995; Niedergang et. al., 2000; Palupi et. al., 2000). This procedure is more difficult to standardize and precise quantitation of ligand receptors/cell is not as accurate as with Scatchard analysis using

20 radiolabeled ligand. This method is more given to comparing binding capacity between two different cell populations. With fluorescent activated cell sorting, it also requires that

the binding surface exist in a monodispersed state capable of being analyzed in the flow cell of that instrument.

4   3.   It is also possible to allow ligand binding to cell surfaces and to then incubate the cells with a fluorescent labeled antibody to the ligand, wash, and analyze cell fluorescence by fluorescent spectrophotometry or <u>fluorescent activated cell sorting</u> (FACS). To assess ligand receptor levels only, one can incubate cells with a fluorescent

8   labeled antibody to the receptor and measure the fluorescence of the cells by fluorescent activated cell sorting (Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999).

4.   Western blotting is a technique where cell lysates obtained by detergent

12   treatment are separated by electrophoresis and the separated components contained within the electrophoresis gel are driven onto a protein-binding membrane via electric current. The membrane with its cell constituents separated by molecular weight is blocked with a non-specific protein and can than be analyzed for particular cellular

16   constituents by treatment with an antibody to that constituent followed by treatment with an enzyme conjugated antibody to the first antibody. Enzyme containing regions of the membrane are detected using color-producing or light-emitting substrates for that enzyme.

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5.   Dot-blotting or slot-blotting is where the cell lysate is applied directly to a binding-membrane without prior separation by electrophoresis. The membrane is

blocked and treated as described in the previous paragraph to detect particular cell constituents. Unlike Western-blotting, the molecular weight of detected material is not ascertained.

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    6. We found that the binding of transferrin to tumor cell surfaces correlated with the aggressiveness of those cells; i.e.: the more metastatic tumor cells bound more transferrin than did poorly metastatic cells (Cavanaugh and Nicolson, 1991, Cavanaugh

8 and Nicolson, 1998; Cavanaugh et. al., 1999). These studies required that we accurately assess the transferrin binding capability of cells in question. Initially, this was performed by examining the ability of the cells to bind ^{125}I-transferrin and the ability of non-labeled transferrin to inhibit that. Dealing with radioactive iodine has many drawbacks including

12 the inherent hazardous nature of the material, its short shelf life, and expensive waste disposal. In searching for novel methods for measuring transferrin binding using non-radioactive procedures, we came upon the discovery that fluorescein-labeled transferrin (or FITC-labeled) would stimulate the growth of cells in culture similarly to native

16 transferrin. We also found that fluorescein-labeled transferrin could be internalized by cells and that this internalization could be competed for by an excess of un-labeled (or native) transferrin. The apparent retention of biological activity by fluorescein-labeled transferrin lent us to examine other technologies available to specifically detect the

20 labeled protein. Many antibody suppliers now sell anti-fluorescein antibodies. These were initially developed to detect fluorescein-labeled oligonucleotides hybridized to sample RNA on Northern blots. These same antibodies can easily detect fluorescein-

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labeled proteins on Western blots (Samuel *et. al.*, 1988). We next assessed as to whether

or not the combination of these reagents together would allow for the detection of

fluorescein-labeled protein bound to cell surfaces. Cells were treated with fluorescein-

4   labeled transferrin with and without an excess of native transferrin. After an appropriate

incubation period, the cells were washed extensively and lysed with a detergent

containing buffer. The lysate components were separated by electrophoresis and

electroblotted onto a nitrocellulose membrane. The membrane was blocked with non-fat

8   dry milk and incubated with a rabbit anti-fluorescein antibody. The membrane was

washed and incubated with goat horse radish peroxidase-conjugated anti-rabbit IgG. The

membrane was washed again and treated with a light emitting (enhanced luminescence)

substrate for horse radish peroxidase. One band at ≈70,000 in molecular weight was seen

12   in all lanes loaded with cells that initially were exposed to fluorescein-labeled transferrin

only. In lanes loaded with cells that had also received an excess of native transferrin, a

markedly reduced band, or no band at all was seen. This method allowed for the

sensitive determination of transferrin binding to cells without the need for radioactively

16   labeled transferrin. Furthermore, the molecular weight of the bound ligand was verified

via the electrophoresis step.

7.   The major difference in the method of this ligand binding method in comparison

20   to those of the referenced patents and literature papers is the final detection method. In

our case, the bound hapten-ligand is detected by immunological means after

solubilization (or cell lysis) and immobilization onto a membrane. In the referenced

cases, bound radio-labeled ligand is detected on solubilized cells by counting; or in the case of fluorescent-labeled ligands, by fluorescent detection of the label on intact cells by optical means such as cytometry.

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BRIEF SUMMARY OF THE INVENTION:

1.   The present invention relates to the need in biological research to measure the ability of cells or other surfaces to bind a given compound (hereafter referred to as a ligand). The ligand could be a growth factor or any other factor whose study involves the need for persons to assess the ability of cells, or any other insoluble particle or material, to bind it. The invention requires that the binding factor be conjugated with an immunogical reactive hapten such as fluorescein and at the same time retain biological and binding activity.

2.   This new ligand binding method ~~The invention~~ makes use of many available anti-hapten antibodies which specifically recognize a hapten-conjugated binding entity or ligand (hapten-ligand) in a complex mixture of other compounds which are naturally devoid of the hapten. The hapten-ligand is presented in excess to the substrate to which it binds. After binding, excess hapten-ligand is washed off, and all bound hapten-ligand is solubilized with or without solubilized substrate components. The solubilized mixture is applied to a membrane support directly or is separated by electrophoresis and then applied to a membrane support. The included membrane-bound hapten-ligand is detected by treatment of the membrane with anti-hapten antibody and then by an enzyme-conjugated-antibody to the anti-hapten antibody. The amount of resultant membrane-associated localized enzyme is determined by incubation with a color or light-producing substrate for that enzyme. For maximum sensitivity, a light-producing substrate is

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applied and the enzyme is detected by enhanced chemi-luminescence.   A series of
known amounts of pure hapten-ligand can be applied to the membrane support, or can be
separated by electrophoresis and applied to the membrane support, and similarly

4   detected, to determine a signal to dose standard curve which can be used to ascertain the
amount of hapten-ligand in the unknowns. Thus, the system lends itself to very precise
and user-defined standardization. The two-antibody incubation steps amplify the signal
so that in combination with enhanced chemi-luminescence, very low levels of hapten-

8   ligand can be detected. When used this way, the system can be used to measure ligand
binding to cell surfaces without the need for radio-labeled ligand. Another feature of the
system is that all of the reagents required are stable and have long shelf-lifes. The system
is a low-cost, non-hazardous, sensitive, non-radioactive, precisely standardized method

12   for determing the binding of compounds to substrates. In particular, the method lends
itself to the measurement of hapten-conjugated protein binding to cell and tissue surfaces.
Specifically, the method has been perfected for the use of measuring fluorescein-
conjugated transferrin, fluorescein-conjugated concanavalin A, fluorescein-conjugated

16   annexin-V, fluorescein-conjugated avidin, and fluorescein-conjugated insulin binding to
tissue culture cell surfaces. This invention not only offers a novel non-radioactive
method for assessing ligand binding to cell surfaces, but can be used to quantitate the
binding of any recognizable hapten-containing binding factor to any surface, providing

20   that the factor can be subsequently removed, (and perhaps separated by electrophoresis;
optional), and bound to a membrane support.

BRIEF DESCRIPTION OF THE DRAWINGS:

Figures 1A-1B show a schematic of the strategy of the assay, illustrating the measurment of the binding of fluorescein labeled transferrin to cell surfaces.

Figures 2A-2C show reproductions of actual enhanced chemiluminescence films of electrophoretically separated unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein labeled transferrin to cell surfaces.

Figures 3A-3B show a schematic of the strategy of the assay, illustrating the measurment of cellular apoptosis by analyzing the binding of fluorescein labeled annexin-V to cell surfaces.

Figures 4A-4C show a reproduction of the actual enhanced chemiluminescence film of electrophoretically separated unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein annexin-V to cell surfaces.

Figures 5A-6=5C show a reproduction of the actual enhanced chemiluminescence film of unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein conjugated concanavalin A to cell surfaces, after separation by electrophoresis.

Figures 6A-6D show a reproduction of the actual enhanced chemiluminescence films of unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein conjugated concanavalin A to cell surfaces by dot blotting, without preliminary separation.

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Figures 7A-7C show a reproduction of the actual enhanced chemiluminescence film

of electrophoretically separated unknowns and standards, and the graphical analysis of

the luminescence data, obtained when analyzing the binding of fluorescein avidin to cell

4   surfaces.

Figure 8 shows a reproduction of the actual enhanced chemiluminescence film of

electrophoretically separated unknowns and standards obtained when analyzing the

binding of fluorescein insulin to cell surfaces.

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~~Figure 1. A.) A schematic of the strategy of the assay. A cell monolayer is exposed to a~~

~~solution of FITC-Tf (1) or FITC-Tf plus an excess of unlabeled Tf (2). In either case, 3~~

~~molecules of Tf bind per cell. When washed and lysed, cells from 1 produced a lysate~~

12   ~~containing 9 molecules of FITC-TF whereas cells from 2 produce a lysate containing 1~~

~~molecule of FITC-Tf. In B.), both samples are electrophoresed and the gels blotted.~~

~~With the cells from 1, 9 molecules of FITC-Tf are present in the 70,000 molecular weight~~

~~region of the blot; when this blot is incubated with rabbit anti-FITC and then with goat~~

16   ~~anti-rabbit IgG-HRP, a large band is seen. With the cells from 2, only one molecule of~~

~~FITC-Tf is present on the blot and a minimal band is seen on the blot after ECL.~~

~~Figure 2. Reproductions of actual enhanced chemiluminescence films obtained when this~~

20   ~~assay was performed (procedures described on pages 8 and 9). (A.) Measurement of~~

~~FITC-transferrin (FITC-Tf) binding to MTLn2/TfR cells. Cells at 50 - 60 % confluency~~

~~growing in 12 well plates were serum-starved, then treated at 4° C with increasing levels~~

of FITC-Tf. After 2h, cells were washed, lysed, and equal cell equivalents were electrophoresed, blotted, incubated with goat anti-FITC, then with anti-goat HRP and an HRP-ECL substrate. The blot was then analyzed using the BIO-RAD fluor-S

4 Multiimager. Lanes 1-6 = lysates from cell exposed to the concentration of FITC-TF listed below the blot. Lanes 7-12 = lysates from cells treated as in 1-6, but also with a 200-fold excess of unconjugated Tf. (B.) Results from a blot treated as in A but loaded with pure FITC-TF standards in the amounts (in ng) indicated on the top. The pure

8 FITC-Tf samples were electrophoresed, blotted, and measured using the two antibodies mentioned in A, followed by ECL. (C.) Results from the quantitation of B using the Bio-Rad Multiimager, indicating the type of standard curves achievable.

12 Figure 3. A.) A schematic of the strategy of the assay when used to detect apoptotic cells. Cells in early apoptosis are known to bind the protein Annexin V whereas normal cells bind little or none of this protein. Cells in apoptosis (1) or normal non-apoptotic cells (2) are exposed to a solution of FITC-Annexin V. When washed and lysed, cells

16 from 1 produced a lysate containing FITC-Annexin V whereas cells from 2 produce a lysate containing no FITC-Annexin V. In B.), both samples are electrophoresed and the gels electro-blotted. With the cells from 1, the FITC-Annexin V molecules are present in the 33,000 molecular weight region of the blot. When this blot is incubated with anti-

20 FITC and then with anti-goat-IgG-HRP, HRP is localized to the 33Kd region of the blot and the HRP-containing bands are detected on X-ray film using an HRP chemiluminesent substrate. This produces a band on the film at 33 Kd. With the cells from 2, no FITC-

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~~Annexin V is present on the blot, the initial antibody and therefore the second antibody~~

~~do not bind, no light is produced upon incubation with an HRP chemiluminesent~~

~~substrate, and no band is seen on the film.~~

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~~**Figure 4.)** Results obtained when this assay was used to measure the binding of FITC-~~

~~Annexin V to rat MTLn3 mammary adenocarcinoma cells, as described in Figure 3. The~~

~~cells were grown to confluence in six well plates. Cells were induced to apoptose by~~

8 ~~treatment with 4 ug/ml Camptothecin (dissolved in DMSO). Controls received DMSO~~

~~only. After 24h, wells were washed three times with and equilibrated in 1 ml binding~~

~~buffer (25 mM HEPES, .15 M NaCl, 2.5 mM CaCl₂, pH 7.5). FITC-Annexin V was~~

~~added to 50 ng/ml and the cells were incubated for 30 min at 25° C. Cells were then~~

12 ~~washed extensively with binding buffer, and lysed in 1 ml of RIPA lysing solution. The~~

~~lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by~~

~~SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Annexin V.~~

~~Separated proteins were blotted onto a nitrocellulose membrane which was blocked and~~

16 ~~then incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP~~

~~containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results~~

~~of quantification of the standards is shown in B. The curve from B was used to calculate~~

~~Annexin V bound by the cells, the results of which are shown in C. The results indicate~~

20 ~~greater Annexin V binding by the camptothecin treated cells.~~

~~**Figure 5.** A.) A schematic of the strategy of the assay when used to detect specific PCR~~

~~products.   biotin-labled PCR products are produced from sample DNA using biotin-~~

~~labeled primers. De-natured products are applied to a well which contains a bound DNA~~

4 ~~probe which is designed to recognize the middle region of the desired PCR product. The~~

~~PCR products bind to the probe, the well is washed of unbound components and the~~

~~hydridized PCR species removed by heat denaturation. B.) The removed products are~~

~~separated by agarose electrophoresis and are blotted to a nytran membrane. The~~

8 ~~membrane is blocked, and incubated with rabbit anti-biotin. The membrane is washed,~~

~~incubated with goat anti-rabbit IgG-HRP, washed again, and HRP containing bands are~~

~~detected using a light-emitting HRP substrate and ECL. Any non-specific PCR products~~

~~which have made it this far can be dis-regarded, as long as they of different molecular~~

12 ~~weight from the desired product.~~

~~**Figure 6.)** Results obtained when this assay was used to measure the binding of FITC-~~

~~Concanavalin A (Con A) to rat MTLn3 mammary adenocarcinoma cells. The cells were~~

16 ~~grown to confluence in six well plates. The growth media was replaced with a binding~~

~~buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as~~

~~a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Con~~

~~A was added to replicate wells so that the final concentrations of FITC-Con A were 0.1,~~

20 ~~1.0, and 10.0 ug/ml. One well of each FITC-Con A concentration also received 200~~

~~ug/ml of native (un-conjugated Con A). The cells were incubated for 2h at 4° C, washed~~

~~extensively with PBS, and lysed in 800 uL of RIPA lysing solution. The lysates were~~

centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Con A. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with

4   rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results of quantification of the standards is shown in B. The curve from B was used to calculate specific Con A bound by the cells, the results of which are shown in C.

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Figure 7.) Results obtained when the samples from figure 5 were analyzed by a dot-blot procedure. For the standards, increasing volumes (2, 4, 8, and 16 uL) of a 100 ng/ml FITC-Con A solution were applied to a nitrocellulose membrane. For the lysates, 4 uL of

12   lysates from cells treated with 0.1, 0.5, and 1.0 ug/ml FITC-Con A (with or without an sxcess native Con A) were applied to the membrane. The membrane was blocked, incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-HRP, and HRP-containing sites detected with ECL (A, B). The dots were quantitated using a Bio-Rad

16   Fluor S imager. Data from the standards (A, C) were used to determine the amount of Con A bound by the cells (B, D). This displays the usefulness of the technique in a dot-blot procedure, where the SDS-PAGE and electroblottin steps are eliminated.

20   Figure 8.) Results obtained when this assay was used to measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The growth media was replaced with a binding buffer

consisting of 25 mM HEPES-buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were

4 0.1, 0.2, and 0.4 ug/ml. The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Avidin. Separated proteins were blotted onto a

8 nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results of quantification of the standards is shown in B. The curve from B was used to calculate specific Con A bound by the cells, the results of

12 which are shown in C.

Figure 9.) Results obtained when this assay was used to measure the binding of FITC-Insulin to human K562 erythroleukemia cells. Logarithmically growing cells in

16 suspension culture were collected by centrifugation and washed twice by suspension in and centrifugation from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM HEPES (pH 7.5). Cells were adjusted to a density of 2×10^6/ml (in binding buffer), and were equilibrated to 4° C. To 1 ml of cell suspension was added 20

20 µl of 1mg/ml FITC-Insulin (in binding buffer; final concentration = 20 µg/ml). An additional tube also received non-conjugated Insulin at a level of 200 µg/ml. Cell

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~~suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were~~

~~collected and washed three times by suspension in and centrifugation from binding~~

~~buffer.   Cell pellets were lysed in 0.4 ml/tube of Schager-Von Jagow (SVJ)~~

4   ~~electrophoresis system treatment solution and treated at 95° C for 5 min. Lysates (100 μl~~

~~each) were separated by SDS-PAGE run according to Schager-Von Jagow along with~~

~~FITC-Insulin standards, and treated aliquots of the cell-unbound incubation mixture. Gel~~

~~components were transferred to nitrocellulose and membrane-associated FITC detected~~

8   ~~as described with other ligands.   In the figure, a scan of the ECL film is shown.~~

~~Increasing signal is returned for increasing loads of FITC-insulin in the standards (lanes~~

~~1-3). The FITC-insulin bound by the cells is easily observed (lane 4), and this is reduced~~

~~significantly when excess un-conjugated insulin was present (lane 5). This procedure~~

12 ~~consistently displays higher molecular weight forms of insulin formed after application to~~

~~cells, perhaps due to the presence of insulin binding proteins (lanes 4-7).~~

<u>DETAILED DESCRIPTION OF THE INVENTION:</u>

1. The object of the present invention is to provide a method for the sensitive non-

4   radioactive assessment of ligand binding to insoluble surfaces. Specifically, the method

developed measures the binding of transferrin, concanavalin-A, avidin, annexin-V, and

insulin to cell surfaces. ~~The basic detailed method using fluorescein-conjugated~~

~~transferrin as a detectable antibody-recognizable hapten tracer follows. Specific~~

8   ~~alterations of this procedure for other ligands are described in the description of figures~~

~~section.~~

2. <u>A schematic of the detection and competitive binding strategy of the assay is</u>

<u>shown in Figure 1. In Figure1A, a cell monolayer is exposed to a solution of fluorescein</u>

12   <u>labeled transferrin (FITC-Tf). In Figure 1B, an identical cell monolayer is exposed to a</u>

<u>solution of FITC-Tf plus an excess of unlabeled transferrin. In either case, 3 molecules</u>

<u>of Tf bind per cell. When washed (Figure 1C) and lysed, cells from Figure 1A produced</u>

<u>a lysate containing 9 molecules of FITC-TF (Figure 1E), whereas cells from Figure 1B</u>

16   <u>produce a lysate containing 1 molecule of FITC-TF (Figure 1D, Figure 1F). In Figure</u>

<u>1G, both samples are loaded onto an electrophoresis gel, along with standards containing</u>

<u>increasing levels of known amounts of FITC-Tf. When electrophoresed, the Tf and cell</u>

<u>lysate proteins stack up and migrate according to their molecular weight (Figure 1H).</u>

20   <u>These are blotted onto a membrane as shown in Figure 1I, where their relative positions</u>

<u>are maintained. The membrane is blocked and treated with goat anti-FITC (Figure 1J),</u>

<u>which specifically binds to the FITC-Tf only. The membrane is washed and treated with</u>

anti-goat IgG-peroxidase, which recognizes only the goat anti-FITC (Figure 1K). The membrane is washed again and treated with an ECL substrate for peroxidase (Figure 1L), where light produced per band correlates with membrane FITC-Tf content per band. The

4 light produced is recorded on an X-ray film (Figure 1M), which is imaged so that each band is assessed for optical density/mm^2 (ODu/mm2; Figure 1N). With the cells from Figure1A, a large band is seen on the X-ray film exposed by this light, at the same vertical position as the standard signals. Thus, the detection of the ligand is seen, at the

8 correct molecular weight for transferrin. The amount of FITC-Tf in this band can be estimated by comparing its signal to that of the standards. The amount of FITC-Tf bound per cell is then calculated, from the cell density of the culture plate used in Figure 1A. When the FITC-Tf band produced from the cells from Figure1B is analyzed, minimal

12 light production is seen. Thus, competition for Tf binding to the cells between FITC-Tf and Tf is seen, demonstrating specific binding to the cells by the FITC-Tf.

3. The basic detailed method using fluorescein-conjugated transferrin as a

16 detectable antibody-recognizable hapten tracer follows:

4. Fluorescein-conjugated iron-saturated (holo) human transferrin was obtained from commercial sources. Cultured cells to be measured were grown to 50 – 60 % confluence in 12 well plates. Cells were incubated with serum-free minimal essential media (alpha

20 modification; $\propto$-MEM) for 12 h and then again with fresh $\propto$-MEM for another 12 h. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a ~~Coulter Counter~~ cell counter. Media in remaining wells was replaced

with 1 ml binding buffer (BB) which consisted of: 25 mM HEPES in α-MEM containing

2mg/ml of bovine serum albumin (BSA); pH 7.5. The cell wells were then allowed to

equilibrate to 4°C in a refrigerator. Sets of 5 replicate wells received increasing amounts

4   of FITC-Tf, from 0.02 to 0.5 0.025 to 0.1 µg/ml final FITC-Tf. Two wells of each FITC-

Tf concentration set then received unlabeled holo human transferrin so that the final

unlabeled [Tf] = Tf concentration was 100 µg/ml. After a 2h incubation at 4°C, all media

was saved (= unbound samples), and the wells were all washed 4 times by the addition

8   and drainage of 1 ml of 4°C phosphate-buffered saline (PBS). All wells then received

0.5 ml of an RIPA cell lysing solution which consisted of PBS containing 1% v/v nonidet

P-40 detergent, 0.5% v/v deoxycholic acid, 0.1% v/v sodium dodecyl sulfate (SDS), 100

µg/ml phenylmethyl sulfonyl chloride, and 0.1 TIU units/ml Aprotinin. Cells were

12   incubated with the lysing solution for 30 min at 4°C and all lysates were pipetted into

separate 1.5 ml conical tubes. The tubes were centrifuged at 5,000 X g for 10 min and

400 µL of each supernatant was transferred to a fresh tube. All of these tubes received

166 µL of a 4X concentrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis

16   (SDS-PAGE) treatment solution (0.5 M Tris, 8% w/v SDS, 2% v/v beta--

mercaptoethanol, 1.0% w/v bromophenol blue, 20% v/v glycerol, pH 6.8), and were

treated at 95°C for 10 minutes. Treated samples (150 uL each) were loaded onto an

acrylamide SDS-PAGE electrophoresis gel. The gels consisted of a 12 X 10 cm

20   separating gel containing 0.375 M Tris, 0.1% w/v SDS, 10 % w/v acrylamide, pH 8.8;

and a 12 X 2 cm stacking gel containing 0.125 M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 6.8.

4   5. ~~Treated samples were loaded onto a 12 X 12 cm 10% acrylamide SDS-PAGE electrophoresis gel (150 uL/sample)~~ Unbound samples were treated similarly to cell lysate samples, and loaded onto the electrophoresis gel. Typically, these have to be diluted 1:10 – 1:100 in 1 X SDS-PAGE treatment solution, prior to electrophoresis, to

8   produce a signal within a readable range. The assay was standardized by loading a series of treated solutions of known amounts of pure FITC-Tf onto the electrophoresis gel. These consisted of 7 samples applied so that 2.4, 4.8, 9.7, 19, 39, 78, 156, and 313 ng FITC-Tf protein were delivered per well, respectivley. All samples were electrophoresed

12   at 40 mA constant current until the dye front was 1 cm from the bottom of the gel. The gel was equilibrated in a transfer buffer of 48 mM Tris, 39 mM glycine. A 14 X 14 cm nitrocellulose membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components

16   were transferred to the membrane at a constant voltage of 40 V for 1.5h.

6. The membrane was blocked at 4°C overnight in a block solution consisting of Tris buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 7.8) containing 0.1% tween 20

20   and 5% w/v non-fat dry milk. The membrane was incubated with 1:1000 rabbit anti-FITC in block solution for 2h at 25°C, and washed three times (20 min each) with 50 ml

TBS. The membrane was incubated with 1:2000 horse radish peroxidase-conjugated goat anti-rabbit IgG in block solution for 2h at 25°C and washed again. ~~Each membrane was covered with an ECL substrate for HRP (Amersham)~~ The membrane was covered with an

4 enhanced chemiluminescent substrate for horse radish peroxidase, was wrapped in plastic, and was loaded into an X-ray film cassette along with an 8 X 10 inch piece of ~~Amersham ECL hyperfilm~~ chemiluminescent-detecting film. The film was developed after 1 min exposure and an additional film was added which was developed after 20 min

8 exposure. The film was scanned, and bands produced on the film were marked and quantitated by measurement of optical density/mm^2 (ODu/mm^2), using a CCD camera equipped imager.

7. Figure 2A displays an image of the film from the transferrin binding method

12 obtained after the 1 min exposure. Here, all lanes were loaded with lysates from equal quantities of cells initially exposed to the concentration of FITC-TF listed above the blot. Signals from duplicate wells are shown. The figure shows that when increasing levels of FITC-Tf are initially present, that higher levels of FITC-Tf bind to a constant amount of

16 cells, which is in keeping with normal binding behavior. In Figure 2B, the left half of the gel was loaded with cells initially treated as in Figure 2A, but also with 100 µg/ml of unconjugated Tf. The figure also displays markedly lower binding of FITC-Tf to the cells when an excess of Tf is initially present, indicating competition between FITC-Tf

20 and Tf for cell binding, and therefore specific binding of FITC-Tf to the cells. The right half of Figure 2B shows results from the analysis of equal amounts of aliquots of the initial unbound samples from Figure 2A.

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8. <u>Figure 2C shows an image of the film obtained when standard solutions of FITC-</u>

<u>Tf were electrophoresed, blotted, and analyzed for FITC content as indicated above.</u>

<u>Here, the amounts of FITC-Tf loaded onto the electrophoresis gel (in ηg) are indicated</u>

4   <u>on the top. Figure 2D displays the standard curve obtained when the density of the bands</u>

<u>obtained from the image analysis of the film from Figure 2C were plotted against the</u>

<u>amount of FITC-Tf present in each band. The equation shown on the curve was used to</u>

<u>calculate the amount of FITC-Tf present in the bands from Figure 2A and 2B, thus</u>

8   <u>enabling the determination of the weight of FITC-Tf bound per cell, and unbound per</u>

<u>well, at each initial FITC-Tf concentration. This data was converted to molecules and</u>

<u>moles, using a Tf molecular weight of 75,000. This data was then plotted as a</u>

<u>conventional Scatchard analysis as shown in Figure 2F, to obtain Tf receptors per cell.</u>

12

9. ~~Unbound samples are run similarly to cell lysate samples. Typically, these have~~

~~to be diluted 1:10 — 1:100 in SDS-PAGE treatment solution prior to electrophoresis, to~~

~~produce a signal within a readable range. The assay is standardized by loading known~~

16   ~~amounts of pure FITC-Tf onto an electrophoresis gel and repeating all of the above~~

~~procedures. The signal returned from the imager is plotted against the amount of FITC-~~

~~Tf contained in the band and a standard curve is constructed to calculate the amounts of~~

~~FITC-Tf bound by the cells.~~

20   . 10. ~~General applications: The assay strategy can apply to any ligand conjugated with~~

~~a compound which can be specifically recognized by an antibody. In particular, anti-~~

~~digoxygenin, anti-rhodamine and anti-biotin antibodies exist which would recognize~~

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~~ligands conjugated with those compounds. The material to which the ligand binds to can~~

~~be other than cells. Any particles or other insoluble material can serve as the binding~~

~~surface. Centrifugation and re-suspension of suspended particulate binding substrates~~

4 ~~would serve as a method for washing those of unbound ligand. The current method~~

~~requires that the bound ligand be removed from the binding surface so that it can be~~

~~separated by electrophoresis. It must also bind to a conventional transfer membrane for~~

~~detection with the antibody. Other specific applications accomplished to date include the~~

8 ~~study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding~~

~~of Avidin to cells, and the study of the binding of Annexin-V to cells. With the latter~~

~~protein, this assay could be utilized to assess cellular apoptosis without the need for a~~

~~FACS analyzer.~~

12   11. The binding of annexin V to cell surfaces has been recognized as an indicator of

early apoptosis (Zhang *et. al.*, 1997). With conventional procedures, cells are removed

from plates, treated with FITC-annexin V, and analyzed by FACS. The removal of cells

from tissue culture plates using conventional trypsin or EDTA reagents can in itself

16 induce cell stress, apoptosis, and cellular annexin V binding (Darzynkiewicz *et. al.*,

1998; LeGall *et. al.*, 2000). Therefore, the conventional use of annexin V binding as a

measure of apoptosis in adherent cells is problematic. In contrast, this new method

would measure the binding of FITC-annexin V to adherent cultured cells *in situ* (Figures

20 3 and 4), where binding and washing occur first, before the cells are removed from plates

for analysis. Therefore, the amount of FITC-annexin V detected would accurately

represent that bound by cells in their natural culture environment. Thus, the method

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outlined in this new method circumvents conventional problems and provides for a more

authentic measure of natural cellular annexin V binding.

4   ~~12. The assay could be used to verify the hybridization of biotin-labeled DNA to~~

~~other DNA molecules. In one scenario, biotin-labeled PCR products are hybridized to an~~

~~immobilized DNA probe which specifically recognizes the desired PCR product (among~~

~~a mixture of non-specific products). After binding, the bound PCR product is released~~

8   ~~through heat de-naturation, is separated by agarose electrophoresis, electro-blotted to~~

~~nytran, and is detected by incubation with species-x anti-biotin followed by incubation~~

~~with anti-species-x-IgG-HRP and ECL. The final result yields a major band at the~~

~~expected bp size of the PCR product. Any non-specific bands of different size can be~~

12   ~~ignored during analysis of the film by an image analyzer (Figure 4). As with the above~~

~~stated protein procedures, the proper molecular weight of the desired product is verified.~~

13. <u>A schematic of the strategy of the assay when used to detect apoptotic cells is</u>

<u>shown in Figure 3. Cells in apoptosis (Figure 3A) or normal non-apoptotic cells (Figure</u>

16   <u>3B) are exposed to a solution of FITC-Annexin V. When washed (Figure 3C) and lysed,</u>

<u>cells from Figure 3A produced a lysate containing FITC-Annexin V (Figure 3E), whereas</u>

<u>cells from Figure 3B produce a lysate containing no FITC-Annexin V (Figure 3D, Figure</u>

<u>3F). In Figure 3G, both samples are loaded onto an electrophoresis gel, along with</u>

20   <u>standards containing increasing levels of known amounts of FITC-Annexin V. When</u>

<u>electrophoresed, the Annexin V and cell lysate proteins stack up and migrate according to</u>

<u>their molecular weight (Figure 1 H). These are blotted onto a membrane as shown in</u>

Figure 3I, where their relative positions are maintained. The membrane is blocked and treated with goat anti-FITC (Figure 3J). This specifically binds to the FITC-Annexin V

4 only. The membrane is washed and treated with peroxidase-anti-goat IgG, which recognizes only the goat anti-FITC (Figure 3K). The membrane is washed again and treated with an ECL substrate for peroxidase (Figure 3L), where light produced per band correlates with membrane FITC-Annexin V quantity per band. The light produced is recorded on an X-ray film (Figure 3M), which is imaged so that each band is assessed for

8 ODu/mm2 (Figure 3N). With the cells from Figure 3A, plentiful FITC-Annexin V binds, this is then present on the blot, the initial antibody and therefore the second antibody bind, light is produced upon incubation with an HRP chemiluminescent substrate, and a band is seen on the film. The amount of FITC-Annexin V in this band can be estimated

12 by comparing its signal to that of the standards. The amount of FITC-Annexin V bound per cell is calculated, from the cell density of the culture plate used in Figure 3A. With the cells from Figure 3B, no FITC-Annexin V binds, none is present on the blot, the initial antibody and therefore the second antibody do not bind, no light is produced upon

16 incubation with an HRP chemiluminescent substrate, and no band is seen on the film.

14. The detailed mehods for the lysis, electrophoresis, blotting, and ECL detection steps for annexin V, concanavalin A, Avidin, and insulin binding assays were the same as those outlined above in detail, for transferrin. The other specifics of these assays follow.

20 15. Figure 4 displays actual results obtained when this assay was used to measure the binding of FITC-Annexin-V to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. Cells were induced to apoptose by treatment

with 4 µg/ml Camptothecin (dissolved in DMSO). Controls received DMSO only. After

24 hours, wells were washed three times with and equilibrated in 1 ml binding buffer (25

mM HEPES, 0.15 M NaCl, 2.5 mM $CaCl_2$, pH 7.5). FITC-Annexin V was added to 50

4 ng/ml and the cells were incubated for 30 min at 25° C. Cells were then washed

extensively with binding buffer, and lysed in 1 ml of RIPA lysing solution. The lysates

were centrifuged at 5,000 X g for 5 min., the supernatants were assessed for total protein,

and equal protein equivalents of the supernatants were treated (by the addition of one

8 third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) for, and

separated by SDS-PAGE. Also run on the same gel were four pure FITC-Annexin V

standards of 0.5, 1, 2, and 4 µg Annexin V protein per well. Separated proteins were

blotted onto a nitrocellulose membrane which was blocked and then incubated with rabbit

12 anti-FITC, and then with goat anti-rabbit IgG-HRP. HRP containing bands were then

detected by ECL. Figure 4A shows a scan of the ECL detection film, with each lane

marked at the top as to the sample applied. Figure 4B displays the standard curve

obtained when the density of the bands obtained from the image analysis of the standards

16 from Figure 4A were plotted against the amount of Annexin-V present in each standard

band. The equation shown on the curve was used to calculate the amount of Annexin-V

present in the bands from the cell lysates, thus enabling the determination of Annexin-V

bound per cell equivalent (or cell protein) for the various treatments.

20   16. To further test and illustrate another embodiment of the assay, the ability of the

method to detect the binding of Concanavalin A (Con A) to cells was examined. Figure 5

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displays the results obtained when this assay was used to measure the binding of FITC-

Con A to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to

confluence in replicate, in six well plates. The cell number in three wells was determined

4   by trypsinization of those cells followed by enumeration on a cell counter. The growth

media of test wells was replaced with a binding buffer consisting of 25 mM HEPES

buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at

pH 7.5. The cultures were taken to 4° C and FITC-Con A was added to replicate wells so

8   that the final concentrations of FITC-Con A were 0.1, 1.0, and 10.0 μg/ml. One well of

each FITC-Con A concentration also received 200 μg/ml of native (un-conjugated Con

A). The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in

800 uL of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min.,

12   and equal cell equivalents of the lysate supernatants were treated for (by the addition of

one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and

separated by SDS-PAGE. Also run on the same gel were four pure FITC-Con A

standards consisting of 1, 2, 4, and 8 ng total FITC-Con A protein loaded per lane,

16   respectively. Separated proteins were blotted onto a nitrocellulose membrane which was

blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP.

HRP containing bands were detected by ECL onto an X-ray film. The film was imaged

to obtain the optical density units/mm^2 of the bands. A scan of the film is shown in Figure

20   5A. Figure 5B displays the standard curve obtained when the density of the bands

obtained from the image analysis of the standards from Figure 5A were plotted against

the amount of FITC-Con A present in each standard band. The equation shown on the

curve was used to calculate the amount of Con A present in the bands from the cell

lysates, thus enabling the determination of Con A bound per cell equivalent for the

4   various treatments, as shown in Figure 5C. Cells which were initially treated with both

FITC-Con A and un-conjugated Con A displayed markedly lower binding of FITC-Con

A than cells which received FITC-Con A only, indicating competition for binding

between FITC-Con A and unconjugated Con A, further indicating specific cell binding

8   by the FITC-Con A.

17. The replacement of electrophoresis with dot-blot techniques is possible. This

would require that the only immune-recognizable conjugated component present prior to

dot-blotting would be the desired product and/or absolutely minimal interaction of either

12   antibody with non-specific sample components

18. Figure 6 displays the results obtained when the samples from Figure 6 were

analyzed by a dot blot procedure. For the standards, increasing volumes (2, 4, 8, and 16

μL) of a 100 ng/ml FITC-Con A solution were applied to a nitrocellulose membrane.

16   For the lysates, 4 μL of lysates from cells treated with 0.1, 0.5, and 1.0 μg/ml FITC Con

A (with or without an excess of native Con A) were applied to the membrane. The

membrane was blocked, incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-

HRP, and HRP-containing sites detected with ECL onto an X-ray film. The scans of the

20   actual films from the standards are shown in Figure 6A, and that for the cell lysates in

Figure 6B. The dots on the film were quantitated using an imager to obtain the optical

density units/mm^2 of each dot. Figure 6C displays the standard curve obtained when the

density of the bands obtained from the image analysis of the standards from Figure 6A were plotted against the amount of Con A present in each standard dot. To maintain linearity, only the first three were used. The equation shown on the curve in Figure 6C

4 was used to calculate the amount of Con A present in the dots from the cell lysates in Figure 6B, thus enabling the determination of Con A bound per cell equivalent for the various treatments, as shown in Figure 6D. As with Figure 5, cells which were initially treated with both FITC-Con A and un-conjugated Con A displayed markedly lower

8 binding of FITC-Con A than cells which received FITC-Con A only, indicating competition for binding between FITC-Con A and unconjugated Con A, further indicating specific cell binding by the FITC-Con A. This displays the usefulness of the technique in a dot-blot procedure, where the SDS-PAGE and electroblotting steps are

12 eliminated.

19. Another experiment designed to test and illustrate the use of the method, was one where the examination of the ability of the method to detect the binding of avidin to cells was conducted. Figures 7A - 7C display the results obtained when this assay was used to

16 measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. The growth media was replaced with a binding buffer consisting of 25 mM HEPES

20 buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were 0.1, 0.2, and 0.4 μg/ml. The cells

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were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 1 ml of RIPA

lysing solution/well. The lysates were centrifuged at 5,000 X g for 5 min., and the

supernatants representing equal cell equivalents were treated (by the addition of one third

4   volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and separated

by SDS-PAGE. Also run on the same gel were four pure FITC-Avidin standards of 100,

200, 400, and 800 ρg total FITC-Avidin protein loaded per lane, respectively. Separated

proteins were blotted onto a nitrocellulose membrane which was blocked and incubated

8   with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP containing bands

were detected by ECL onto an X-ray film. The film was imaged to obtain the optical

density/mm^2 (ODu/mm^2) of the bands. A scan of the film is shown in Figure 7A. Figure

7B displays the standard curve obtained when the density of the bands obtained from the

12   image analysis of the standards from Figure 7A were plotted against the amount of FITC-

·Avidin present in each standard band. The equation shown on the curve was used to

calculate the amount of FITC-Avidin present in the bands from the cell lysates, thus

enabling the determination of FITC-Avidin bound per cell equivalent for the various

16   treatments, as shown in Figure 7C.

       20. To further test the versatility of the method, its ability to detect the cellular

binding of the low molecular weight protein insulin was examined. Figure 8 displays the

results obtained when this assay was used to measure the binding of FITC-Insulin to

20   human K562 erythroleukemia cells. Logarithmically growing cells in suspension culture

were collected by centrifugation and washed twice by suspension in and centrifugation

from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM

HEPES (pH 7.5). Cells were adjusted to a density of 2×10^6/ml (in binding buffer), and

were equilibrated to 4° C. To 1 ml of cell suspension was added 20 μl of 1 mg/ml

4   FITC-Insulin (in binding buffer; final concentration in cell suspension = 20 μg/ml). An

additional tube also received non-conjugated Insulin at a level of 200 μg/ml. Cell

suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were

collected and washed three times by suspension in and centrifugation from binding

8   buffer. The initial supernatants were kept as the unbound samples. Cell pellets were

lysed in 0.4 ml/tube of Schagger-Von Jagow (SVJ) electrophoresis system treatment

solution (50 mM Tris-HCl, 2% w/v SDS , 1% v/v beta-mercaptoethanol, 5% v/v glycerol,

0.1% w/v bromophenol blue, pH 6.8), and treated at 95° C for 5 min. Unbound samples

12 were likewise treated by the addition of one third volume of a 4X concentrate of the

sample treatment solution, and exposure to 95° C for 5 min. Lysates (100 μl each) and

aliquots of the treated unbound samples were separated by SDS-PAGE run according to

Schagger-Von Jagow. These gels consisted of a 12 X 10 cm separating gel containing

16 1M Tris, 0.1% w/v SDS, 12% w/v acrylamide, pH 8.45; and a 12 X 2 cm stacking gel

containing 0.75M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 8.45. Gels were run in an

electrode buffer of 0.1M Tris, 0.1M Tricine, 0.1 % SDS. Also run on the same gel were

three treated pure FITC-Insulin standards of 1, 2, and 4 ng total FITC-Insulin protein

20 loaded per lane, respectively. Gel components were transferred to a nitrocellulose

membrane which was blocked and incubated with rabbit anti-FITC and then with goat

anti-rabbit IgG-HRP. HRP containing bands were detected by ECL onto an X-ray film. In the Figure 8, a scan of the X-ray film is shown. The lanes of the membrane are shown at the top, and the samples that those lanes received are shown at the bottom. Increasing

4   signal is returned for increasing loads of FITC-insulin in the standards (Figure 8, lanes 1-3). The FITC-insulin bound by the cells is easily observed (Figure 8, lane 4), and this is reduced significantly when excess un-conjugated insulin was present (Figure 8, lane 5). This procedure consistently displays higher molecular weight forms of insulin formed

8   after application to cells, perhaps due to the presence of insulin binding proteins (Figure 8, lanes 4-7).

21. General applications: The assay strategy can apply to any ligand conjugated with a compound which can be specifically recognized by an antibody. In particular, anti-

12   digoxygenin, anti-rhodamine and anti-biotin antibodies exist which would recognize ligands conjugated with those compounds. The material to which the ligand binds to can be other than cells. Any particles or other insoluble material can serve as the binding surface. Centrifugation and re-suspension of suspended particulate binding substrates

16   would serve as a method for washing those of unbound ligand. The current method requires that the bound ligand be removed from the binding surface so that it can be separated by electrophoresis. It must also bind to a conventional transfer membrane for detection with the antibody. Other specific applications accomplished to date include the

20   study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding of Avidin to cells, the study of the binding of Annexin V to cells, and the study of the

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binding of insulin to cells. With the Annexin V protein, this assay could be utilized to

assess cellular apoptosis without the need for a FACS analyzer.

22. In another embodiment, the assay could be used to verify the hybridization of of a

4   known biotin-labeled DNA to a surface. After binding, the bound labeled DNA is

released through heat de-naturation, is separated by agarose electrophoresis, electro-

blotted to nytran, and is detected by incubation with species-x anti-biotin followed by

incubation with anti-species-x IgG-HRP and ECL. The final result yields a major band at

8   the expected bp size of the labeled DNA. As with the above stated protein procedures,

the proper molecular weight of the desired product is verified by comparison to standards

of the labeled DNA run on the same electrophoresis gel.

12

23. Conclusion: this new method is a procedure for measuring the binding of an entity

(ligand) to a surface by using a hapten-conjugated version of the ligand (hapten-ligand),

where the hapten is recognizable by an antibody. An excess of the hapten-ligand is

16   presented to the binding surface and excess (unbound) hapten-ligand is washed off.

Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or

separated by electrophoresis and applied to a membrane support. Known quantities of,

or standards of the hapten-ligand are also applied to a membrane support or separated by

20   electrophoresis and applied to a membrane support. The membrane-bound hapten-ligand

is detected by application of an enzyme-conjugated antibody to the hapten; or by

application of an antibody to the hapten followed by application of an enzyme-conjugated

antibody to the anti-hapten antibody. The resultant membrane-associated enzyme is detected and quantitated by the application of a color or light-producing substrate which reacts with the enzyme. <u>Results obtained from the standards are used to construct a</u>

4   <u>standard curve which is then used to calculate the amount of hapten-ligand in the membrane areas corresponding to the unknowns.</u> Thus, the amount of hapten-ligand originally bound to the surface can be determined. This assay method has the advantages of providing verification of the molecular weight of the binding substance (ligand) via the

8   electrophoresis step. It eliminates the need for radioactive materials. The procedure provides for high sensitivity detection as the dual antibody incubation steps amplify the signal significantly. The procedure allows for easy standardization as different user-definable levels of a standard solution of the Hapten-ligand can be simultaneously

12   applied to the electrophoresis gel or to the dot-blot or slot-blot membrane

16

ABSTRACT OF THE DISCLOSURE:

The invention is a procedure for measuring the binding of an entity (ligand) to a

4 surface by using a hapten-conjugated version of the ligand (hapten-ligand). An excess of

the hapten-ligand is presented to the binding surface and excess (unbound) hapten-ligand

is washed off. Bound hapten-ligand is then solubilized (removed) and applied to a

membrane support or separated by electrophoresis and applied to a membrane support.

8 Standard amounts of hapten-ligand are similarly applied to the membrane. The

membrane-bound hapten-ligand is detected by application of an enzyme-conjugated

antibody to the hapten; or by application of an antibody to the hapten followed by

application of an enzyme-conjugated antibody to the anti-hapten antibody. The resultant

12 membrane-associated enzyme is detected and quantitated by the application of a color or

light-producing substrate which reacts with the enzyme. Results obtained from the

standards are used to calculate the amount of hapten-ligand in the membrane areas

corresponding to the unknowns. Thus, the amount of hapten-ligand originally bound to

16 the surface can be determined. A combination of the use of anti-hapten antibodies along

with membrane-blotting technologies to assess hapten-ligand binding to surfaces is not

found in the scientific or patent literature, particularly in regards to assessing protein

binding to cell surfaces.

20